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GENETIC ANALYSIS OF THREE BARK BEETLE SPECIES FROM PONDEROSA PINE FORESTS OF ARIZONA

GENETIC ANALYSIS OF THREE BARK BEETLE SPECIES FROM PONDEROSA PINE FORESTS OF ARIZONA

By Christopher J. H. Allender

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ABSTRACT

In ponderosa pine (*Pinus ponderosa* Laws.) forests of Arizona, bark beetles (*Coleoptera: Curculionidae: Scolytinae*) play an important role as native disturbance agents that help to stimulate natural forest succession. These phloephagous insects accelerate forest disturbance, nutrient cycling, and snag (i.e, dead tree) recruitment by reducing tree vigor and causing host mortality. These actions are thought to have positive influences on forest resilience and diversity by promoting different class sizes of trees, overall rejuvenation of the forests, and habitat for a wide variety of organisms. However, from 2001-2003 elevated bark beetle activity has caused unprecedented levels of ponderosa pine mortality. To provide improved management strategies, it is essential to develop a better understanding of the population structure of the bark beetle species in order to facilitate analysis of their dispersal patterns. Of the aggressive species causing ponderosa pine mortality, *Ips pini* (Say), *Dendroctonus brevicomis* LeConte, and *D. frontalis* Zimmermann are recognized as the most economically and ecologically important and require further investigation.

Here, I use fluorescently-labeled Amplified Fragment Length Polymorphism (fAFLP) analysis to investigate the population structure of the above three species along an elevation gradient in north-central Arizona. This technique uses randomly

distributed polymorphic loci to generate high-resolution genetic fingerprints suitable for population level analyses. My specific goals were to i) develop genetic markers that identify genetic variation among and within populations of *I. pini*, *D. brevicomis*, and *D. frontalis*, and ii) examine population structure within each species to better understand their dispersal patterns across a portion of their range in northern and central Arizona.

I generated genetic fingerprints for more than 500 beetle specimens and analyzed their genetic diversity. Utilizing non-metric multidimensional scaling (NMDS) ordination, I determined that fAFLP analysis provides powerful markers for detecting genetic variation among the three examined bark beetle species. For all species, gene flow estimates using analysis of molecular variance (AMOVA) were high, suggesting a lack of differentiation and great dispersal among sampling locations. Furthermore, it was difficult to discern population subdivision within each species by finding differences among sampling locations and elevations using NMDS ordinations and analysis of similarity (ANOSIM) estimates. These findings suggest that outbreaks of *I. pini*, *D. brevicomis*, and *D. frontalis* probably occur at regional rather than local scales because of the ability of the bark beetles to rapidly disperse over large distances.

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As with any research project, there were many people that helped with the data collection and technical knowledge. A special thanks to the AFLP gurus Jim Schupp, Scott Woolbright, Gery Allan, Barb Honchak, Nashelly Meneses, and Melanie Hadlock for their assistance with the grueling task of teaching me how to run AFLP analysis from all aspects of DNA extraction to data analysis. I also want to thank the field and lab crews from the forestry department (Northern Arizona University, NAU) and the Rocky Mountain Research Station (RMRS) who were responsible for the weekly sampling and identification of the bark beetles used in this research. These individuals included Chris Hayes, Andy Miller, Kelly Williams and others.

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On a personal note, my family and friends are the people that helped me stay sane and determined while completing this research. Although many of the names mentioned above fall into this category as well, I want to recognize a few more individuals for their support along the way. Of course, a very special thank you goes to my wife who has offered unconditional encouragement and support during my ups and downs of grad school life. A general "thank you for being there" to the people I feel closest: Lisette, Dan, Chris, Jason, Neal, and Lisa. I especially have a great appreciation for the new friends I have made since beginning grad school. This crew includes Diana, Tatum, Doug, Becky, Ray, and Matt. And finally to my parents, you have been my biggest supporters and I hope to successfully launch soon!

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PREFACE

This thesis document contains two chapters. The first is an introductory section including some background information on the importance of bark beetles in forest ecosystems and a description of each species in this study. The second is a manuscript chapter formatted for submission to Environmental Entomology. Co-authors include Karen M. Clancy, Thomas E. DeGomez, Joel D. McMillin, Scott A. Woolbright, Paul Keim, and David M. Wagner. It includes all parts of a typical journal article which may include some information that is redundant of the first chapter. Each chapter contains a separate literature cited.

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CHAPTER 1

INTRODUCTION

Bark beetle ecology/biology.

Bark Beetles (*Coleoptera: Curculionidae: Scolytinae*) are common phloephagous insects of coniferous forests. Over 6,000 species are known to exist worldwide (Byers 1995) and most conifer species in North America are affected by at least one type of bark beetle (Wood 1982). Since some species cause tree mortality, the opposite objective for most timber management goals, over the past century bark beetles have been considered to be destructive pests (Miller and Keen 1960, Furniss and Carolin 1977, Haack and Byler 1993). However, they are now believed to have positive influences on forest sustainability and resilience by accelerating essential disturbance and nutrient cycling (Furniss and Carolin 1977, Kolb et al. 1994, Samman and Logan 2000, Kipfmueller et al. 2002). Basically, by inducing tree and stand mortality, bark beetles provide habitat and naturally drive forest succession for organisms that depend on transitional areas of forests (Amman 1977, Stone and Wolfe 1996). Along with causing tree mortality, bark beetles promote growth and spread of bacteria and fungi that greatly increase the availability of nutrients and organic matter in soils (Samman and Logan 2000). All of the above activities help to promote different class sizes of trees and overall rejuvenation of the forests inhabited by these important tree parasites.

Bark beetles form intricate associations with many organisms in their natural environments including plants, animals, and fungi. Each species associated with bark beetles plays an important role for either their survival or population control. Several

studies indicate that bark beetles and their tree hosts have coevolved over the past 200 million years (Sturgeon and Mitton 1982, Wood 1982, Raffa and Berryman 1987). Their roles of initiating gap formation and snag (i.e., dead tree) recruitment during endemic population levels are important for the survival of many other animal and plant species (Stone and Wolfe 1996, Samman and Logan 2000, Kipfmueller et al. 2002). In fact, with some systems, interactions between the beetles and their associated fungi are known to accelerate tree mortality (Wood 1982, Christiansen et al. 1987, Paine et al. 1997). These combined actions help to overcome tree defenses more effectively than the actions of the beetles alone (Wood and Bright 1987).

Bark beetles have a great influence over forest ecosystems because of their methods of survival. With the exception of adult bark beetle flights, every part of their life cycle is completed within the cambium or bark of trees (Furniss and Carolin 1977, Wood 1982). Adult female beetles lay eggs in elaborate egg galleries where the larva hatch and feed on surrounding tissues (Wood 1982). The actual feeding behaviors differ among species but all utilize the phloem tissue and, when in high numbers, effectively girdle their host trees by creating tunnels underneath the bark (Furniss and Carolin 1977, Wood 1982). This results in reduced tree vigor by compromising the trees' ability to transport water and nutrients through their boles. Once the infestation becomes too large for the trees to fight off invasion, they eventually dehydrate and die (Furniss and Carolin 1977, Cates and Alexander 1982, Raffa and Berryman 1983).

In addition to their roles as disturbance agents, bark beetles serve as an important prey source to a number of predators, including an array of insect and bird

species (Samman and Logan 2000, Kipfmueller et al. 2002). Several studies have shown that some insect predators partially control bark beetle populations and that these events may follow cyclic population patterns (Turchin et al. 1999). However, none of these predators have demonstrated the ability to prevent bark beetle outbreaks (Reeve 1997, Turchin et al. 1999).

As insect predators many clerid beetle (*Coleoptera: Cleridae*) species specialize in eating bark beetles and their larva (Reeve 1997). Some of these predator species track bark beetles using the various pheromones that the bark beetles produce and volatiles given off by the colonized trees (Herms et al. 1991). These predators attack bark beetles on the exterior of the bark and lay their eggs within the crevices of the bark or bark chips (Cronin et al. 2000). Upon hatching, their larva search for and attack bark beetle larva in the egg galleries created by the bark beetles (Cronin et al. 2000).

In addition to insect predators, many birds are also known to feed on bark beetles. Most common is predation by woodpeckers. In many pine forests it is common to encounter a bark beetle-infested tree that has had its' bark stripped off, which is indicative of woodpecker activity (DeMars and Roettgering 1982). They remove the bark with their beaks while searching for adult bark beetles and their larva.

Individual bark beetle species vary in their biology, which assists with identification. These differences include variation in physical appearance, host species preference, time for completion of life cycles, reproductive behaviors, construction of egg galleries, attack strategies, and fungal associates (Wood 1982).

Morphology of adult bark beetles is the most common method for species identification, with the best distinguishing characteristics found on the head, thorax, and elytra (Wood 1982). Regarding host preference, there are both specialists, which attack just one host species, and generalists, which attack multiple host species. Although host species can be quite diverse, most bark beetles parasitize pine (*Pinus* spp.), spruce (*Picea* spp.), and fir (*Abies* spp.) trees (Wood 1982). In addition, the ability of a species to complete its life cycle is greatly affected by temperature constraints. Because of this, intricate relationships with latitude and elevation have been discovered for a number of taxa (DeMars and Roettgering 1982, Wood 1982).

Bark beetle species also vary in their strategies of invasion. Some species begin their attacks from the top of the tree whereas others initiate attacks from the bottom (Wood 1982). Also, some species specialize in attacking small diameter boles and even branches, whereas others require very large diameter bole sizes to live successfully (Wood 1982, Samman and Logan 2000, Steed and Wagner 2004). In forest ecosystems that support multiple bark beetle species on a single tree species, it is quite common to have several species attack the same tree because of these different ecological niches. Depending on the species, either a male or female invades the host tree initially and expels a pheromone signal that attracts the other sex (DeMars and Roettgering 1982, Wood 1982, Cognato et al. 1999).

In regards to reproductive behaviors, some species are considered to be monogamous (e.g., *Dendroctonus* spp.) whereas others exhibit polygamy (e.g., *Ips* spp.) (Wood 1982). Following successful reproduction, extensive egg galleries are constructed, the pattern of which are usually specific to genera or species (DeMars

and Roettgering 1982, Wood 1982, Samman and Logan 2000). They can be constructed with or against the grain of the wood with some species exhibiting a mixture of both (Wood 1982).

Members of the genera *Dendroctonus* and *Ips* are the dominant bark beetle species in coniferous forests of North America (Furniss and Carolin 1977). Of the 17 *Dendroctonus* and 25 *Ips* species present throughout this region (Wood 1982), only a few are recognized as aggressive species because of their ability to cause tree mortality. Most of these aggressive species have specific fungal associates that provide them with essential nutrition and aid in decreasing tree vigor once inoculated into the woody tissues of trees (Klepzig et al. 1996, Paine et al. 1997, Klepzig et al. 2001). In some cases, examination of beetles and their fungal associates have revealed obligate relationships (Paine et al. 1997, Klepzig et al. 2001, Hofstetter et al. 2005). In fact, most research has been conducted on these aggressive species, with the result that much information is available about their life histories (Coulson 1979, Wood 1982, Berryman et al. 1985, Raffa and Berryman 1987, Byers 1995).

Collectively, *Dendroctonus* species are considered the most destructive biotic agent within their native ranges (Wood 1982). During endemic population levels, aggressive *Dendroctonus* species (southern pine beetle, *D. frontalis* Zimmermann; mountain pine beetle, *D. poderosae* Hopkins; western pine beetle, *D. brevicomis* LeConte; and Douglas fir beetle, *D. pseudotsugae* Hopkins) are thought to be primary invaders that attack healthy, mature trees and act cooperatively with pheromone communication and mass attack to overcome tree defenses (Miller and Keen 1960, Coulson 1979, Borden 1982, Cates and Alexander 1982, Wood 1982, Berryman et al.

1985, Samman and Logan 2000). Most of these species are associated with attacking larger diameter trees.

Ips species are also known for their extremely destructive effects, especially following drought events (DeGomez 2003, Kenaley 2004). They are characterized as secondary invaders because of their tendency to colonize weakened, dead, or dying trees during endemic population levels (Furniss and Carolin 1977, Wood 1982, Raffa and Berryman 1983, Parker 1991, Kegley et al. 1997, Klepzig et al. 2001). It has also been common for these species to increase greatly in number by utilizing slash and windthrow (Kennedy 1969, Samman and Logan 2000, Steed and Wagner 2004). However, it is important to note that during epidemic outbreaks any of the aggressive *Ips* species are capable of killing live trees (Coulson 1979, Borden 1982, Sturgeon and Mitton 1982, Raffa and Berryman 1983, Berryman et al. 1985, Raffa and Berryman 1987).

In the southwestern United States, ponderosa pine (*Pinus ponderosa* Douglas ex. Lawson) is a dominant conifer species that has been associated with a high amount of bark beetle diversity (Wood 1982, Oliver and Ryker 1990, Sanchez-Martinez and Wagner 2002). Ponderosa pine ranges from mid to high elevations (1500 to 2900 m) and is important for timber production and wildlife habitat (Oliver and Ryker 1990). Therefore, it is crucial to closely examine its bark beetle parasites.

The American Southwest is where the distributions of many bark beetle species overlap. Bark beetle species from northern regions such as Alaska and Canada overlap with species that have mostly southern distributions throughout Mexico and Central America (Wood 1982). Although there are many species within

this region, only a few aggressive species cause extensive tree mortality. Of these, *Ips pini* (Say)(pine engraver), *D. brevicomis*, and *D. frontalis* are considered among the most economically and ecologically important and require further investigation (Furniss and Carolin 1977, DeMars and Roettgering 1982, Christiansen et al. 1987, Kegley et al. 1997).

Description of I. pini

I. pini, the pine engraver, is distributed throughout North America and is recognized as a moderate to highly aggressive species (Furniss and Carolin 1977, Wood 1982, Cognato et al. 1999). Its pine hosts are diverse and include *Pinus banksiana* (jack pine), *P. contorta* (lodgepole pine), *P. jeffereyi* (Jeffery pine), *P. ponderosa* (ponderosa pine), and *P. strombus* (eastern white pine), with only *P. ponderosa* being present in the southwestern U.S (Wood 1982, Oliver and Ryker 1990). As with most other *Ips* species, *I. pini* has been observed colonizing dead or dying trees of smaller diameter (Furniss and Carolin 1977, Wood 1982, Raffa and Berryman 1983, Parker 1991, Kegley et al. 1997) but is capable of killing healthy, large diameter trees during epidemic outbreak events (Kennedy 1969, Furniss and Carolin 1977, Parker 1991, Kegley et al. 1997). Also, *I. pini* beetles commonly attack trees after successful colonization by *Dendroctonus* species and have been associated with a major portion of the ponderosa pine mortality recorded during the recent (2001-2003) bark beetle outbreak events in Arizona (Wood 1982, DeGomez 2003).

I. pini beetles are polygamous; a male first invades a host tree (Furniss and Carolin 1977) and usually up to three females follow (Wood 1982). As a result, their

egg galleries are typically Y-shaped and relatively long when compared to other *Ips* species (Wood 1982, Kegley et al. 1997). Some males have been reported to attract up to seven females, which causes a star shape appearance to their egg galleries (Furniss and Carolin 1977). It has been reported that up to five generations per year are observed for *I. pini* in its most southern range (Cognato et al. 1999).

Previous studies have demonstrated distinct lineages of *I. pini* occurring throughout their range in the U.S. Initial studies identified pheromone races based on differential response and production of pheromones between eastern and western populations (Seybold et al. 1995). This difference has been explained by an isolation of the races during the Pleistocene, when glacial events forced *I. pini* populations into separate refugia (Cognato et al. 1999). The races followed the historical biogeography of *P. banksiana* for the eastern populations and *P. contorta* for the western populations (Cognato et al. 1999). In addition, genetic analyses using mtDNA from geographically diverse populations throughout North America identified three distinct lineages that were labeled as eastern, rocky mountain, and western varieties (Cognato et al. 1999). In that study, eastern and western lineages were both present in Arizona, although most individuals were identified as the western type.

Description of D. brevicomis

D. brevicomis, the western pine beetle, is a highly aggressive species distributed throughout the intermountain west of North America (DeMars and Roettgering 1982). Its range spans from British Columbia, Canada to Chihuahua, Mexico (Wood 1982). This species is notorious for causing tree mortality and is

thought to have killed more trees throughout North America than any other organism in recorded history (Wood 1982). This primary invading species prefers to attack healthy, mature *P. ponderosa* and *P. coulteri* (Coulter pine) trees, although it is known to invade other *Pinus* species during outbreak events (DeMars and Roettgering 1982, Wood 1982). *D. brevicomis* beetles tend to invade trees from the bottom and migrate upwards as the invasion progresses. During endemic population levels they tend to attack single trees, whereas epidemic levels are associated with group attacks (Wood 1982). The female is first to invade with a male usually following after the female burrows through the cambium and reaches the phloem tissue (DeMars and Roettgering 1982). As they are monogamous, egg galleries consist of an entrance hole attached to winding and crisscrossing tunnels that extend more laterally than longitudinally (Wood 1982). It is not uncommon to have multiple breeding pairs in one gallery because of their overlapping tunnels. In the southern portion of its range, this species can complete its life cycle in as little as two months and be active year round, especially in years with mild winters (DeMars and Roettgering 1982).

Genetic analysis of *D. brevicomis* has revealed possible cryptic speciation within its range in North America. Based on mtDNA analysis, eastern and western cryptic species distributions were identified, which revealed a close association of the eastern type with the *P. ponderosa* variety *scopulorum* and the western type with the *P. ponderosa* variety *ponderosa* (Kelley et al. 1999). The genetic divergence between the two cryptic species was described as being as high as the diversity previously detected between sister species of *Dendroctonus* (Kelley et al. 1999).

Description of D. frontalis

D. frontalis, the southern pine beetle, is recognized as a primary invading species and occurs in the southwestern and eastern U.S., as well as in Honduras (Wood 1982). Its host trees are very diverse, including: *Pinus echinata* (shortleaf pine), *P. engelmanni* (Apache pine), *P. glabra* (spruce pine), *P. oocarpa* (Ocote pine), *P. palustris* (longleaf pine), *P. ponderosa*, *P. rigida* (pitch pine), *P. strombus*, *P. taeda* (loblolly pine), and *P. virginiana* (Virginia pine) (Wood 1982). Described as close relatives to *D. brevicomis*, they share similar invasion and ecological strategies although they differ enough to allow for coexistence (Wood 1982). This species is also known for invading healthy, mature trees and it tends to invade from the top down (Wood 1982). Again, the female invades first with the male following after she reaches the phloem, although their egg galleries are typically more longitudinal than their relatives. Also, this species is marked by the ability to travel considerable distances to colonize new trees (Wood 1982).

Of the *Dendroctonus*, *D. frontalis* has been intensively studied for its relationships with associated fungi and mite interactions among its host range of *P. taeda* (Klepzig et al. 2001, Lombardero et al. 2003, Hofstetter et al. 2005). Many of the fungal associates have been characterized as exhibiting mutualistic, antagonistic, or passive affiliations (Hofstetter et al. 2005). In general, parasitic mites of *D. frontalis* serve as vectors of their antagonistic fungal associates, which the mites depend on for a food source (Hofstetter et al. 2005).

Bark beetle outbreaks in North America

In North America, bark beetles serve as important indicators of forest health (Logan et al. 2003). In recent years, there has been an increasing amount of large-scale tree mortality due to bark beetle activity throughout many areas in the mountain west. In fact, there has been more tree mortality in the past few years than previously observed since European human colonization (Samman and Logan 2000, Logan et al. 2003). These changes have led many researchers to conclude that many forested lands are not as healthy and resistant to disturbance factors, such as insect infestations and fire, as they once were (Sanchez-Martinez and Wagner 2002). Also, much research has focused on the causes of the suspected forest decline with different conclusions for different geographic areas.

In the northern portions of North America, some bark beetle species have successfully expanded their ranges into areas where they were not present before, or at least not present in such high population levels (Logan et al. 2003). These changes in distribution and abundance have been attributed to a warming trend observed throughout Canada and Alaska (Logan et al. 2003). It has been argued that a period of mild winters has allowed some bark beetle species to live in areas that were originally too cold for their survival (Logan et al. 2003). Since these forest lands have been naïve to this degree of bark beetle activity, they are not well adapted to combat infestation and have experienced large-scale tree mortality.

In the southern portions of the intermountain west, a combination of fire suppression and drought followed by a very moist period over the past century has been blamed for reducing forest health (Samman and Logan 2000, Sanchez-Martinez

and Wagner 2002). In extreme cases, these conditions have led to even-aged forests where trees are in close proximity and therefore have difficulty obtaining water and nutrient resources needed to combat natural disturbance agents (Samman and Logan 2000, Sanchez-Martinez and Wagner 2002). These vast forested areas have exhibited reduced resistance to bark beetle invasion, which has led to many bark beetle outbreaks and catastrophic wildfires. Since there are many factors that contribute to bark beetle outbreaks, it is difficult to pinpoint the exact causes of a specific event and many researchers believe that current forest conditions were at least partly influenced by anthropogenic effects (Sanchez-Martinez and Wagner 2002, Logan et al. 2003).

Recent bark beetle epidemics in Arizona

Between 2001 and 2003, Arizona experienced unprecedented levels of pine mortality due to drought and subsequent bark beetle activity (DeGomez 2003, DeGomez et al. 2006, Gaylord et al. 2006). Over that time period, it was estimated that more than 800,000 hectares of forested land were affected by bark beetles (DeGomez et al. 2006). The highest amount of mortality was observed in 2002, which followed the greatest intensity drought since the mid 1990s (DeGomez et al. 2006). To put this pine mortality into prospective, during 2002 Arizona also experienced its largest wildfire on record, the Rodeo-Chediski fire, which burned about 202,000 hectares. In that same year, bark beetles caused pine mortality on the same amount of land area throughout other areas of Arizona. It was estimated that two million ponderosa pines were killed with some lower elevation areas experiencing complete

ponderosa pine mortality (DeGomez et al. 2006). Some of the greatest tree mortality was observed in the ponderosa and pinyon-juniper forests located within the Coconino, Gila, Kaibab, Prescott, and Tonto National Forests. The forested lands located within the sky island areas of the Coronado National Forest experienced high spruce and fir mortality as well. Since bark beetles typically did not exhibit epidemic population levels in Arizona over the past century (Gaylord et al. 2006), much speculation and research has gone into the causes and control of these recent events. Although no successful landscape-level treatments have been developed, a few methods for saving prize trees or small stands, which use either pheromones or pesticides, have been described (DeGomez et al. 2006).

Objectives of this study

To provide improved management strategies for bark beetles in Arizona, it is important to obtain a better understanding of their movements and dispersal patterns across the landscape. Previous research on bark beetles in Arizona has largely focused on information pertaining to relative species abundances and their interactions with host species. Although a few studies have assessed relationships among bark beetles using genetics, they only identified beetle specimens to species and none have completed a genetics-based population study of bark beetles in Arizona.

Since it is important to assess the population structure of bark beetles in Arizona, the objective of this study was to use genetic techniques to identify bark beetles to species and ask questions regarding the movements and dispersal patterns

of their populations. Specifically, my research objectives were to: 1) develop genetic markers that can be used to detect genetic variation among and within the bark beetle species; 2) assess population subdivisions within each species using beetle specimens collected from multiple geographically distinct locations; and 3) comment on any dispersal or movement patterns detected by statistical analyses throughout the different sampling locations.

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CHAPTER 2

Genetic Analysis of Three Bark Beetle (*Coleoptera: Curculionidae: Scolytinae*) Species from Ponderosa Pine Forests of Arizona.

Abstract. In ponderosa pine (*Pinus ponderosa* Laws.) forests of Arizona, bark beetles play an important role as disturbance agents that help stimulate natural forest succession. However, from 2001-2003 elevated bark beetle activity caused unprecedented levels of ponderosa pine mortality. To provide improved management strategies, it is essential to develop a better understanding of the population structure of these species to facilitate analysis of their dispersal patterns. Here, we use fluorescently-labeled Amplified Fragment Length Polymorphism (fAFLP) analysis to resolve genetic variation among and within sampling locations of *Ips pini* (Say), *Dendroctonus brevicomis* LeConte, and *D. frontalis* Zimmermann. We generated genetic fingerprints for more than 500 beetle specimens and analyzed their genetic diversity. For all species, gene flow estimates among sampling locations were high and population subdivision was difficult to discern. Also, a weak relationship was detected with *I. pini* and elevation. These findings suggest that outbreaks of bark beetles probably occur at regional rather than local scales because of their ability to rapidly disperse over large distances.

Keywords: *Dendroctonus*, *Ips*, AFLP

In the western United States most bark beetles (*Coleoptera: Curculionidae: Scolytinae*) species are native phloephagus insects of coniferous forests that help stimulate forest disturbance, nutrient cycling, and snag (i.e., dead tree) recruitment (Wood 1982, Stone and Wolfe 1996, Samman and Logan 2000, Kipfmueller et al. 2002). These insects utilize the phloem tissue just under the cambium or bark of many pine (*Pinus* spp.), spruce (*Picea* spp.), and fir (*Abies* spp.) trees to complete their life cycles and form extensive egg galleries (Wood 1982). Bark beetles can increase overall forest resilience and diversity by driving forest succession, thus creating habitat for a wide variety of organisms, and by providing an important food source for many species of insects and birds (Kolb et al. 1994, Samman and Logan 2000, Kipfmueller et al. 2002).

Although the diversity of bark beetles that inhabit ponderosa pine (*Pinus ponderosa* Laws.) forests is quite high, only a few species induce tree mortality and are characterized as aggressive. These include primary invaders such as *Dendroctonus* spp., which attack healthy mature trees using pheromone communication and mass attack (Miller and Keen 1960, Coulson 1979, Borden 1982, Cates and Alexander 1982, Wood 1982, Berryman et al. 1985, Samman and Logan 2000), and secondary invaders such as *Ips* spp., which colonize weakened, dead, or dying trees (Furniss and Carolin 1977, Wood 1982, Raffa and Berryman 1983, Parker 1991, Kegley et al. 1997, Klepzig et al. 2001). It is important to note, however, that epidemic population levels of any aggressive species are capable of killing live trees (Coulson 1979, Borden 1982, Sturgeon and Mitton 1982, Raffa and Berryman 1983, Berryman et al. 1985, Raffa and Berryman 1987).

In Arizona, recent outbreaks of *Ips pini* (Say) (pine engraver), *Dendroctonus brevicomis* LeConte (western pine beetle), and *D. frontalis* Zimmermann (southern pine beetle) have drastically altered large areas of ponderosa pine forests. From 2001-2003, a combination of drought and existing high stand density facilitated increased bark beetle activity that has caused unprecedented levels of ponderosa pine mortality in central and northern Arizona. For example, it was estimated that millions of trees in this region were killed by bark beetles (DeGomez 2003, DeGomez et al. 2006). Because of this record high tree mortality, it is essential to develop a better understanding of bark beetle population structure in order to address questions about their dispersal and movements across the landscape.

Here, we use fluorescently-labeled Amplified Fragment Length Polymorphism (fAFLP) analysis to investigate the population structure of *I. pini*, *D. brevicomis*, and *D. frontalis* along an elevation gradient in north-central Arizona. This technique employs randomly distributed polymorphic loci to generate high-resolution genetic fingerprints suitable for population level analyses (see Travis et al. 1996, Keim et al. 1997, Garcia et al. 2002, Paupy et al. 2004). Our specific goals were i) to develop genetic markers that distinguish among and within sampling locations of *I. pini*, *D. brevicomis*, and *D. frontalis*; and ii) to examine the population structure of these species to better understand their dispersal across a portion of their range in northern and central Arizona.

Materials and Methods

Sample Collection

Adult specimens of *I. pini*, *D. brevicomis*, and *D. frontalis* were collected from 30 sampling locations in north-central Arizona as part of a large elevation gradient study during 2004. A subset of this collection was used for genetic evaluation from only 15 of the 30 sampling locations (Fig. 1, Table 1). Table 1 lists site information, including dates of collection and elevation. The elevation gradient followed one of three designations: high (2,591 ± 76 m), mid (2,134 ± 76 m), or low (1,676 ± 76 m) elevation. At each site, two pheromone-baited funnel traps were used for collection. One trap was baited for *D. brevicomis* using frontalin, exo-brevicomin, and myrcene, which also trapped *D. frontalis*. The other trap was baited for *I. pini* with a combination of lanierone and +03/-97 ipsdienol. Only those collections spanning 36 consecutive days or less were used, in an effort to control for multiple generations. Beetles were sorted and identified to species using morphological character examinations (Wood 1982). Whole beetles were then stored at -20°C until DNA extraction. DNA extraction of whole beetles was completed using a bead milling technique in conjunction with 96-well DNeasy® Tissue Kits (Qiagen, Valencia, CA) as described by Allender et al. (2004). Final elution steps were modified to incorporate two washes of the DNA column with 60 µl of elution buffer such that the final volume contained genomic DNA in 120 µl.

fAFLP procedure

DNA fingerprints were obtained for each sample using fAFLP analysis. We followed the general procedure of Vos et al. (1995) with the following modifications. Restriction-ligation (R-L) reactions were completed using 20 µl of DNA template, 1x RL buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 Ug/ml BSA), and 5 U of both *Eco*I and *Mse*I in a total volume of 50 µl. Samples were incubated for at least 1 h at 37°C before adding the additional 10 µl of ligation reagents (1x RL buffer, 0.12 mM ATP, 1 U T_4 DNA ligase, 1 uM *Eco* adapter mix, and 5 uM *Mse* adapter mix). Another 2 h incubation was performed at room temperature (between 20 and 25°C), followed by a 1:10 dilution of the R-L template in molecular grade water. Pre-selective polymerase chain reaction (PCR) was performed in 50 µl reaction volumes containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 75 ng *Eco*I primer with adenine as the selective nucleotide, 75 ng of *Mse*I primer with cytosine as the selective nucleotide, 1 U of Taq polymerase, and 5 µl of R-L template. The cycling parameters started with an initial 2 min at 72°C, followed by 25 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C. The final step was a 30 min elongation step at 60°C. Samples were then diluted 1:10 in molecular grade water.

The second selective PCR utilized 5' fluorescently-labeled *Eco* primers and unlabeled *Mse* primers. The following four primer combinations were used on all samples: 6FAM-*Eco*AAC-*Mse*CCA, 6FAM-*Eco*AAC-*Mse*CGA, HEX-*Eco*ACG-*Mse*CCA, and HEX-*Eco*ACG-*Mse*CCA. Using 3 µl of diluted products from above, the second selective PCR was performed in 20 µl reaction volumes containing 1x

PCR buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 2% of volume HiDi Formamide (Applied Biosysyems, Foster City, CA), 1 U Taq polymerase, 0.05 μM fluorescently-labeled selective *Eco*I primer, and 0.25 μM selective *Mse*I primer. The PCR cycling parameters described in Travis et al. (1996) were used except the final cycle was repeated an additional 20 times for a total of 30 cycles. Capillary gel electrophoresis and data analysis was completed using a 3730xl DNA sequencer (Applied Biosystems, Foster City, CA) and Genemapper 3.7 software (Applied Biosystems, Foster City, CA).

Genetic analyses

Genetic variation among species was evaluated using non-metric multidimensional scaling (NMDS) ordination. This method is similar to principle components analysis by providing a visual representation but makes fewer assumptions about the type of data and relationships among the samples, while still conserving rank distance relationships in low-dimensional ordination space (Clarke and Warwick 2001). In addition, by applying analysis of similarity (ANOSIM; Clarke 1993), an analog of ANOVA, statistical inference can be applied to the similarity distance rankings created by the ordination (Clarke and Warwick 2001). The NMDS ordinations and ANOSIM analyses were completed using the software program DECODA (Minchin 1991). ANOSIM analyses used 1,000 permutations to generate R [$R = (\textit{mean ranks between groups} - \textit{mean ranks within groups}) / (N(N-1)/4)$] and p values. Bonferroni corrections were applied to subsequent post-hoc analyses based upon a global alpha level of 0.05.

An estimate for overall mean, pair-wise genetic distance was utilized to compare the relative amount of genetic diversity within each species. These distance estimates were compared with one another to determine each species' overall genetic diversity. Genetic distances were computed using the MEGA 3.0 software (Kumar et al. 2004).

We used analysis of molecular variance (AMOVA) and NMDS ordination to assess population structure patterns within each species. This combination allowed us to examine gene flow patterns (AMOVA) and resolve possible sampling location differences (NMDS ordination). AMOVA uses Φ, an analog of F statistics (Weir and Cockerham 1984), to partition the total variance into hierarchical levels of subdivision (Excoffier et al. 1992). We used the software package Genetic Analysis in Excel (GenAlEx) version 6 (Peakall and Smouse 2006) to partition genetic variation among elevations, among sites within elevations, and within sites. NMDS ordinations and ANOSIM analyses were then used to examine if beetles collected from a given sampling location, or from the same elevation band, formed distinct genetic groups. When applicable (i.e., significant global ANOSIM test), post-hoc pairwise comparisons were used to examine differences among specific populations.

Results

Every beetle we examined had a unique fAFLP signature and exhibited an average of 30 markers present per individual. Using the four primer combinations, a total of 333 markers were detected with 255 being polymorphic among our samples. Of these 255 polymorphic markers, 161, 138, and 133 were polymorphic within *I.*

pini, *D. brevicomis*, and *D. frontalis*, respectively. The number of polymorphic markers yielded by each primer combination was 55, 58, 66, and 76. Also, an overall error estimate of 2.6% was determined using a sample of nine replicates from each species across the four primer combinations.

The results for assessing genetic variation among species using NMDS ordination (Fig. 2) indicated separation of the three species into three separate clusters. It was further supported by the ANOSIM test ($R = 0.9998$, $P < 0.001$). Pairwise comparisons revealed that each of the three clusters present in the NMDS ordination (Fig. 2) were statistically different from the others.

The overall mean genetic distances were low and similar for the three species. The result for *I. pini* was the highest (0.104) indicating that it contained the greatest amount of diversity of the three species. It was followed by *D. brevicomis* at 0.093 and then *D. frontalis* at 0.086.

The AMOVA and NMDS ordination results for *I. pini* indicated that some population structure is evident. AMOVA (Table 2A) indicated that variation among elevations, among sites within elevations, and within sites was 1.4, 2.4, and 96.2%, respectively. The NMDS ordinations and ANOSIM analyses for sampling locations (Fig. 3A) and elevation (Fig. 4A) demonstrated significant differences among both sites ($R = 0.0936$, $P < 0.001$) and elevations ($R = 0.0458$, $P = 0.0030$). Post-hoc analysis of sampling locations (Table 3A) indicated that some of the sites significantly differed from one another but without clear patterns. In addition, post-hoc analysis of elevations revealed that the low elevation beetles formed a distinct

group that was significantly different from mid and high elevation specimens (Fig. 4A).

Population structure for *D. brevicomis* was revealed by its AMOVA and NMDS ordination results. The AMOVA (Table 2B) results indicated that variation among elevations, among sites within elevations, and within sites was 0, 5.2, and 94.8%, respectively. The NMDS ordinations for sampling locations (Fig. 3B) and elevations (Fig. 4B) indicated high diversity among samples but no clear groupings. ANOSIM results indicated significant differences among sampling locations (R = 0.1390, $P < 0.001$) and no differences among elevations ($R = 0.0337$, $P = 0.1210$). Subsequent post-hoc analysis of sites (Table 3B) revealed that site 9 differed from all the others except site 3.

Population structure for *D. frontalis* was also evident by its AMOVA and NMDS ordination results. Variation among elevations, among sites within elevations, and within sites was 0, 8.5, and 91.5%, respectively (Table 2C). The NMDS ordination for sampling locations (Fig. 3C) and elevation (Fig. 4C) indicated some sample diversity with population subdivision evident. As with *D. brevicomis*, ANOSIM tests detected significant differences only among sampling locations (R = 0.2526, $P < 0.001$) and not among elevations ($R = 0.0044$, $P = 0.3870$). Further post-hoc analysis of sites (Table 3C) revealed that only site 13 was different from all other locations.

Discussion

Among species genetic variation

We conclude that fAFLP analysis provides powerful markers for detecting genetic variation among the three examined bark beetle species. The high level of differentiation observed in the overall NMDS ordination (Fig 2) and subsequent ANOSIM results suggest these species have very different genetic signatures, which agrees with their current classification as distinct species (Wood 1982).

Since each beetle was assigned to species prior to genetic analysis, we were able to test the reliability of routine morphological classification versus genetic evaluation. We found that only 11 of the total 511 beetles were inconsistent between morphology and the NMDS ordination. After checking the identities of these individuals, we found they were from one collection site and collected on the same date; these discrepancies were only detected between the two *Dendroctonus* species. To further evaluate these beetles for species determination, we sequenced a portion of their cytochrome oxidase 1 genes and found them to be consistent with the fAFLP data (Allender et al., unpublished). These results suggest that the beetles were incorrectly labeled by morphology, and that the overall error rate of the morphological classification method was 2% in this study. In addition, the sequenced regions were determined to be the same as found in previous scientific literature (Kelley and Farrell 1998, Cognato and Sperling 2000). As such, no new sequences were submitted to Genbank.

Genetic variation within species

With respect to genetic variation within species, we conclude that fAFLP analysis suggests there was high genetic homogeneity among sampling locations and that all sites may be part of a large regional (e.g., north-central Arizona) population. The overall mean genetic distance results were suggestive of little differentiation with estimates that were low and similar for all three species. Also, there was difficulty in discerning clear population subdivision patterns using AMOVA (Table 2) and NMDS ordinations (Figs. 3 and 4) indicating a lack of genetic differentiation and high gene flow among sites and elevations.

We attribute the genetic homogeneity among sampling locations to the moderate to strong dispersal capabilities of these species (Cognato et al. 1999). Because our results indicate high gene flow and limited population structure among our sampling locations, we postulate that the scale of our study area was well within the dispersal capabilities of the three bark beetle species. Our results are consistent with previous work showing that strongly dispersing phytophagous insects exhibit homogenizing effects because of elevated gene flow over hundreds of kilometers (Peterson and Denno 1998). The greatest distance within this study area, between the most northern and southern sites, was approximately 120 km (Fig. 1).

Because of the sampling locations utilized in this study, we were able to test for the effects of elevation with respect to gene flow within each species. Elevation has been thought to play an important role in limiting bark beetle species distributions (Wood 1982, Lombardero et al. 2000). However, only *I. pini* indicated an effect of elevation on gene flow. AMOVA results (Table 2A) demonstrated that a weak (1.4%

of variation) but significant ($P < 0.01$) proportion of genetic variance within this species was linked to the three elevation designations. In addition, the NMDS ordinations for elevation (Fig. 4) and subsequent ANOSIM testing only indicated significant differences among elevations for *I. pini*, which agreed with the AMOVA results.

Genetic analysis of *I. pini* revealed interesting population structure patterns. This species demonstrated the greatest amount of differentiation of the three as indicated by its overall mean genetic distance. This higher relative diversity may be a result of reduced dispersal capabilities, previous bottleneck events that lowered the genetic diversity of the other two species, or other selection pressures influencing their population diversity. In addition, the NMDS ordinations (Figs. 3A and 4A) detected two clusters which demonstrate distinct population structure. These results are consistent with a previous study that observed two distinct genetic lineages of *I. pini* in Arizona (Cognato et al. 1999). Even though population subdivision was detected for *I. pini*, we conclude that there must have been high gene flow among the sampling locations because the AMOVA results (Table 2A) indicated that ≈96% of the genetic variation was within sites with only ≈1% due to differences among elevations and ≈2% from differences among sites within elevations.

Genetic analysis of *D. brevicomis* suggests that this species has great dispersal capabilities. AMOVA results (Table 2B) indicated that ≈95% of the genetic variation in this species was within sites with only ≈5% due to differences among sites, which strongly suggests that it had high gene flow throughout its range. Analysis of population subdivision using NMDS ordinations (Figs. 3B and 4B) revealed that *D.*

brevicomis demonstrated the weakest population differentiation patterns observed for the three species and was further supported by the few number of significant differences detected among sites with the pair-wise comparisons (Table 3B). We believe this was due to the close proximity of the sampling locations because there was less geographic distance, approximately 70 km, between the most northern (site 3) and southern (site 11) collection sites (Fig. 1). In fact, only site 9 was significantly different from other sampling locations (Table 3B) and it is important to note that this site was not the most geographically separated (Fig. 1). The reason for this pattern remains unclear.

Genetic analysis of *D. frontalis* suggests it also has wide dispersal among the sampling locations. The AMOVA results (Table 2C) were consistent with the other two species indicating that most of the variation detected was within (≈92%) rather than among (≈9%) sites, which is suggestive of high gene flow. However, *D. frontalis* demonstrated the most variation among sites of the three species. These results also were supported by the NMDS ordination for sampling locations (Fig. 3C) and pairwise comparisons (Table 3C), which detected significant differences between many of the sampling locations. This differentiation was present even though the overall mean genetic distance was the lowest (0.086) of the three species and the NMDS ordination for elevation (Fig. 4C) indicated no significant differences. Furthermore, only site 13 was determined to be significantly different from all other sampling locations and was the most geographically isolated.

Implications for bark beetle management

The lack of genetic differentiation detected within each species is consistent with large dispersal distances and large population size, now and in the past. This means that regional management is needed because our results imply that outbreaks of *I. pini*, *D. brevicomis*, and *D. frontalis* will have an impact over the entire region, not just in the areas where ponderosa pine mortality is high. It also suggests that the recent outbreaks may not have increased the population sizes by that much.

In addition, the genetic differentiation patterns detected for *I. pini* and *D. brevicomis* raise some concerns. For instance, the NMDS ordinations for *I. pini* (Figs. 3A and 4A) showed two possible subpopulations. Because of research demonstrating allopatric speciation leading to cryptic species within *Scolytinae* (Kelley et al. 1999) and previous discussions of three distinct lineages within *I. pini* (Cognato et al. 1999), future experimentation would have to determine whether the two subpopulations of *I. pini* differ enough in their biology (e.g., aggregation pheromones) to change their responses with chemical based treatments (i.e. pheromones, pesticides, etc). Even closely related bark beetle species can have very different aggregation pheromones (Sturgeon and Mitton 1982). Similarly, *D. brevicomis* also showed some separation in its NMDS ordinations (Fig. 3B and 4B) into what might be two different subpopulations. Although we were unable to detect distinct population differences with our genetic analyses, there is evidence for cryptic speciation in *D. brevicomis* (Kelley et al. 1999).

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Table 1. Sampling location information

Map ID no.[a]	Site Name	Elevation Category[b]	Elevation (m)	Sample size (Collection Dates[c])		
				I. pini	*D. brevicomis*	*D. frontalis*
1	Newman Hill	High	2,532	20 (21 May)	18 (21 May)	23 (18 June)
2	Crowley Peak	High	2,507	23 (21 May)	--	--
3	Saddle Mountain	High	2,590	--	15 (28 May)	--
4	Freidlein Prairie	High	2,564	--	13 (28 May)	--
5	Orion Springs	High	2,627	--	--	14 (25 June)
6	Sunset Crater	Mid	2,155	23 (17 June)	18 (17 June)	--
7	Cinder Hills	Mid	2,146	22 (3 June)	21 (17 June)	24 (28 May)
8	Marshall Lake	Mid	2,195	--	23 (27 May)	15 (3 June)
9	Ashurst Lake	Mid	2,164	23 (17 June)	21 (3 June)	--
10	Rim	Mid	2,160	22 (9 June)	--	--
11	Cow Hill	Mid	2,208	--	21 (10 June)	21 (3 June)
12	Moore Creek	Low	1,735	23 (9 June)	--	21 (16 June)
13	Tonto Creek	Low	1,683	21 (2 June)	--	22 (16 June)

Map ID no.[a]	Site Name	Elevation Category[b]	Elevation (m)	Sample size (Collection Dates[c])		
				I. pini	*D. brevicomis*	*D. frontalis*
14	Christopher Creek	Low	1,734	22 (9 June)	--	--
15	Haigler Creek	Low	1,619	22 (9 June)	--	--
Total				221	150	140

[a] See Fig. 1.
[b] See text.
[c] All collections were made in 2004.

Table 2. AMOVA results for *I.* pini (A), *D. brevicomis* (B), and *D. frontalis* (C)

Source	df	SS	% of variation	*P*
A. *I. pini*				
Among elevations	2	64.9	1.4	<0.01
Among sites, within elevations	7	138.3	2.4	<0.01
Within sites	211	2,677.1	96.2	<0.01
B. *D. brevicomis*				
Among elevations	1	19.5	0	0.790
Among sites, within elevations	6	137.7	5.2	<0.01
Within sites	142	1,592.5	94.8	<0.01
C. *D. frontalis*				
Among elevations	2	45.5	0	1.000
Among sites, within elevations	4	115.4	8.5	<0.01
Within sites	133	1,359.0	91.5	<0.01

Table 3. Mean pair-wise genetic distances between sampling locations of *I. pini* (A), *D. brevicomis* (B), and *D. frontalis* (C)

	Map	Map ID no.[a] and elevation category													
Elevation	ID	High							Mid				Low		
category	no.[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A. *I. pini* site comparisons															
High	2	0.097		--	--	--			--			--			
	6	0.109	0.113	--	--	--			--			--			
Mid	7	0.110	0.113*	--	--	--	0.117		--			--			
	9	0.107	0.111	--	--	--	0.124	0.118	--			--			
	10	0.089	0.096	--	--	--	0.107	0.107*	--	0.105		--			
Low	12	0.106*	0.110*	--	--	--	0.120	0.121*	--	0.117	0.106*	--			
	13	0.093*	0.098*	--	--	--	0.101*	0.111*	--	0.108*	0.092*	--	0.099		
	14	0.104*	0.108*	--	--	--	0.064	0.115*	--	0.120*	0.101*	--	0.114	0.095*	
	15	0.106*	0.110	--	--	--	0.065	0.114	--	0.120	0.102	--	0.116	0.097*	0.059
B. *D. brevicomis* site comparisons															
High	3	0.090	--			--					--	--	--	--	--
	4	0.094	--	0.092		--					--	--	--	--	--

	Map	Map ID no.[a] and elevation category													
Elevation	ID	High							Mid				Low		
category	no.[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mid	6	0.102	--	0.099	0.103	--					--	--	--	--	--
	7	0.096	--	0.096	0.098	--	0.100				--	--	--	--	--
	8	0.092	--	0.091	0.095	--	0.102	0.097			--	--	--	--	--
	9	0.088*	--	0.085	0.091*	--	0.101*	0.096*	0.087*		--	--	--	--	--
	11	0.087	--	0.086	0.091	--	0.096	0.092	0.087	0.086*	--	--	--	--	--
C. *D. frontalis* site comparisons															
High	5	0.083	--	--	--		--			--	--			--	--
Mid	7	0.088*	--	--	--	0.093	--			--	--			--	--
	8	0.081*	--	--	--	0.083	--	0.088*		--	--			--	--
	11	0.089*	--	--	--	0.090	--	0.098*	0.079	--	--			--	--
Low	12	0.078*	--	--	--	0.080*	--	0.085*	0.063	--	--	0.075*		--	--
	13	0.086*	--	--	--	0.094*	--	0.100*	0.095*	--	--	0.097*	0.093*	--	--

An * denotes significant differences between populations using ANOSIM pairwise population comparisons with Bonferonni correction (global $\alpha < 0.05$).

[a] See Fig. 1 and Table 1.

Fig. 1. Map of sampling locations in north-central Arizona. Unique identifiers for each site are indicated with numbers 1-15 (see Table 1). Green to dark brown color gradient corresponds to lower to higher elevations.

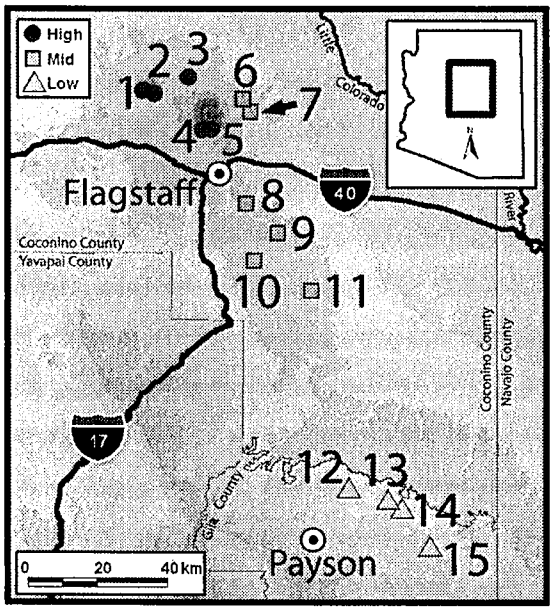


Fig. 2. NMDS ordination plot of three bark beetle species using 255 polymorphic AFLP markers.

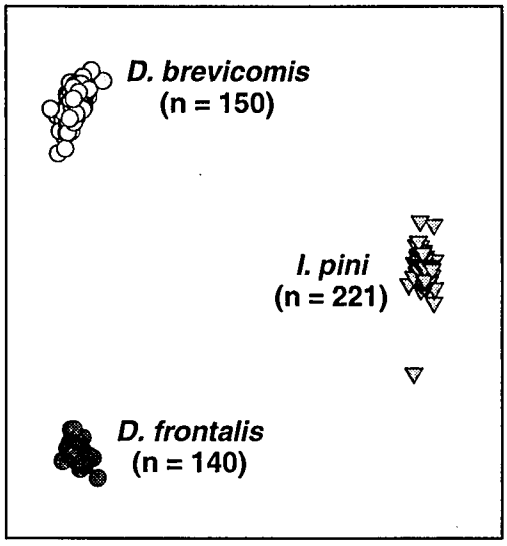
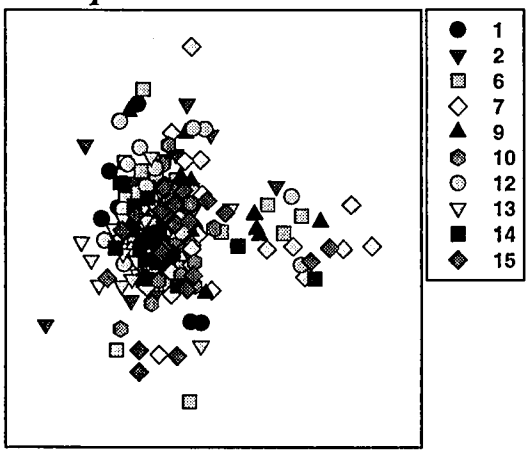
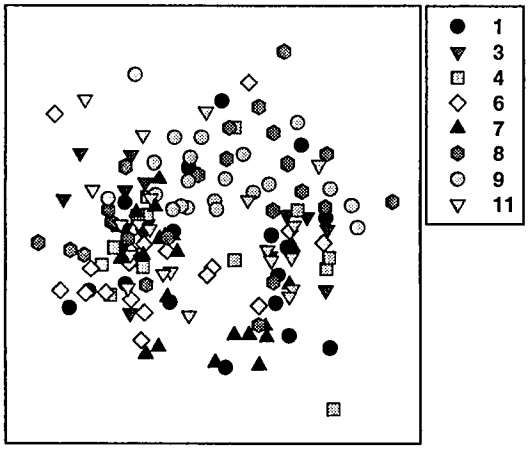


Fig. 3. NMDS ordination plots of sampling locations for *I. pini* (A), *D. brevicomis* (B), and *D. frontalis* (C). Numbers refer to specific sampling locations (see Fig. 1 and Table 1). An * indicates sites that significantly differed from all others using ANOSIM pair-wise estimates (see Table 3).

A. *I. pini*



B. *D. brevicomis*



C. *D. frontalis*

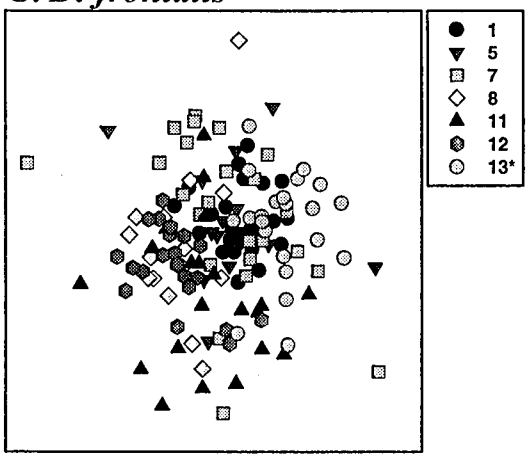
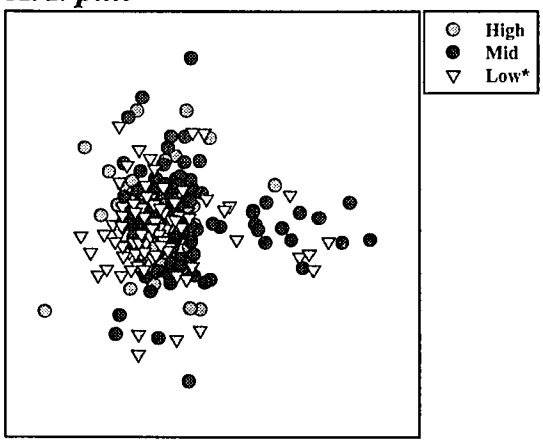
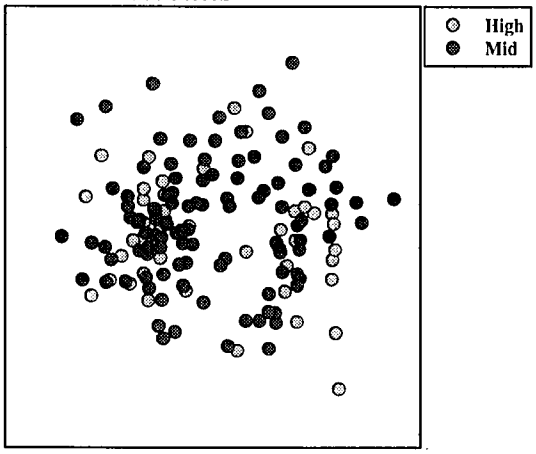


Fig. 4. NMDS ordination plots of elevation for *I. pini* (A), *D. brevicomis* (B), and *D. frontalis* (C). High, mid, and low refer to elevation designations (see text and Table 1). An * indicates significant differences using ANOSIM.

A. *I. pini*



B. *D. brevicomis*



C. *D. frontalis*